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Dietary *Capsicum* and *Curcuma longa* oleoresins increase intestinal microbiome and necrotic enteritis in three commercial broiler breeds

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ABSTRACT

Three commercial broiler breeds were fed from hatch with a diet supplemented with *Capsicum* and *Curcuma longa* oleoresins, and co-infected with *Eimeria maxima* and *Clostridium perfringens* to induce necrotic enteritis (NE). Pyrotag deep sequencing of bacterial 16S rRNA showed that gut microbiota compositions were quite distinct depending on the broiler breed type. In the absence of oleoresin diet, the number of operational taxonomic units (OTUs), was decreased in infected Cobb, and increased in Ross and Hubbard, compared with the uninfected. In the absence of oleoresin diet, all chicken breeds had a decreased *Candidatus* Arthromitus, while the proportion of *Lactobacillus* was increased in Cobb, but decreased in Hubbard and Ross. Oleoresin supplementation of infected chickens increased OTUs in Cobb and Ross, but decreased OTUs in Hubbard, compared with unsupplemented/infected controls. Oleoresin supplementation of infected Cobb and Hubbard was associated with an increased percentage of gut *Lactobacillus* and decreased *Selenihalanaerobacter*, while Ross had a decreased fraction of *Lactobacillus* and increased *Selenihalanaerobacter*, *Clostridium*, *Calothrix*, and *Geitlerinema*. These results suggest that dietary *Capsicum*/*Curcuma* oleoresins reduced the negative consequences of NE on body weight and intestinal lesion, in part, through alteration of the gut microbiome in 3 commercial broiler breeds.

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1. Introduction

Avian necrotic enteritis (NE) is an intestinal disease caused by *Clostridium perfringens* and represents one of the most economically important infectious diseases affecting the world's poultry industry (Shirley and Lillehoj, 2012; Williams, 2005). Over the past several decades, prophylactic use of in-feed antibiotics has had a major impact on controlling the incidence of NE. However, regulatory and voluntary restrictions on the use of antibiotics in poultry have led to a re-emergence of NE during commercial poultry production (Songer, 1996; Williams, 2005). Alternatives to antibiotics that have shown promise in controlling avian NE include prebiotics, probiotics, symbiotics, plant extracts, feed enzymes, bacteriophage, bacterial lysins and antimicrobial peptides (Engberg et al., 2012; Hong et al., 2012; Jozefiak et al., 2013; Lee et al., 2013; Li et al., 2012; Millet and Maertens, 2011; Seal et al., 2013; Timbermont et al., 2010; Wu et al., 2014).

Modulation of innate immunity using natural foods and herbal products offers another avenue to enhance poultry health and reduce the negative effects of pathogen infection (Lillehoj and Lee, 2012). Phytonutrients, when given as feed additives, stimulate the chicken immune system and enhance host defense against intestinal bacteria and parasites (Cheng et al., 2014; Lee et al., 2013; Lillehoj et al., 2011; Naidoo et al., 2008; Siragusa et al., 2008; Sultan et al., 2014). *Capsicum annuum* (pepper), *Curcuma longa* (turmeric), *Lentinus edodes* (shiitake mushroom), *Carthamus tinctorius* (safflower), and *Cinnamomum cassia* (cinnamaldehyde) all enhance innate and acquired immunities to *Eimeria* avian coccidiosis and NE (Lee et al., 2010, 2011). Dietary feeding of a naturally occurring mixture of oils and resins (oleoresins) from *C. annuum* and *C. longa* augmented resistance to experimental NE in commercial Ross broilers (Lee et al., 2013). At the genetic level, transcriptional changes in chicken genes mediating innate immunity were associated with dietary feeding of this oleoresin to Ross birds (Kim et al., 2010; Lee et al., 2013). Since birds were infected with *Eimeria maxima* on day 14 post-hatch followed by infected *C. perfringens* on

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day 18, intestinal change and bird performance caused by NE were observed on day 20 (Jang et al., 2013). We previously reported that Cobb chickens exhibited both greater body weight loss and intestinal lesions compared with Ross and Hubbard chickens using an experimental model of *C. perfringens*/*E. maxima* co-infection to replicate natural NE on day 20 (Jang et al., 2013). These results suggested that the Cobb breed may be more susceptible to NE compared with the other 2 broiler lines.

Recent development and application of next-generation sequencing technologies using 16S rRNA gene have allowed investigating the significant roles of microbiota in the gastrointestinal tract and facilitated the investigation of host–pathogen interaction and the role of the intestinal microbiota in diseases (Callaway et al., 2009; Hume et al., 2011). Inflammation in the intestine is often associated with alteration of intestinal microbiota homeostasis (Wu et al., 2014). Several studies reported that mixed *Eimeria* infection causes an alteration of intestinal microbiota community in broilers fed dietary supplement such as essential oils, probiotics or dietary fish meals (Humer et al., 2014; Molnar et al., 2015; Wu et al., 2014). However, there have been no reports to observe the distribution of gut bacterial communities and identification of specific OTU before/after feeding dietary phytonutrients in 3 commercial broiler breeds to an infectious NE challenge with *E. maxima*/*C. perfringens*. Therefore, the current study was undertaken to determine whether dietary supplementation with a mixture of oleoresins from *Capsicum*/*Curcuma longa* might potentiate NE disease resistance in Cobb, Hubbard, and/or Ross chickens, and if so, to elucidate the intestinal microbiota of 3 commercial broilers in depth by using the high-throughput pyrosequencing and provide a better insight into the microbial communities and diversity in 3 groups; 1) unsupplemented and uninfected (control), 2) unsupplemented and *E. maxima*/*C. perfringens* co-infected (NE), 3) *Capsicum*/*Curcuma longa* oleoresin supplemented and co-infected (NE + XT). This is the first study to show significant dietary effect of phytonutrients (*C. annuum* and *C. longa*) on genetically selected commercial broiler breeds to NE and associated with intestinal microbiota.

2. Materials and methods

2.1. Experimental animals and diets

One-day-old male Cobb 500, Ross 308, and Hubbard broilers were housed in Petersime starter brooder units and provided with feed and water *ad libitum*. All birds were maintained in a temperature-controlled environment at 30 °C for days 1 and 2 post-hatch followed by gradual reduction to 23 °C until the end of the experiment. Chickens (5 per group, 15 per breed) were randomly assigned to one of three treatment groups: unsupplemented and uninfected (control), unsupplemented and *E. maxima*/*C. perfringens* co-infected (NE), or *Capsicum*/*Curcuma longa* oleoresin supplemented and co-infected (NE + XT). Two additional treatment groups, unsupplemented chickens infected with either *C. perfringens* (CP) alone or *E. maxima* (EM) alone, were included and observed, but not included in the results. Dietary supplementation consisted of 4 mg of each oleoresin per kg of feed (100 ppm XTRACT® Nature, Pancosma, Geneva, Switzerland). Chickens were infected with *E. maxima* strain 41A (1.0×10^4 oocysts/bird) by oral gavage on day 14 post-hatch followed by oral gavage with *C. perfringens* strain Del-1 (1.0×10^9 colony forming units/bird) isolated from a commercial poultry flock with endemic NE on day 18 (Fig. 1) (Jang et al., 2013; Lee et al., 2013). Uninfected birds received an equal volume of PBS by oral gavage. To facilitate development of NE, the chickens were fed an antibiotic-free certified organic starter diet containing 17% crude protein and 61% carbohydrate between days 1 and 14 post-hatch and a standard grower diet containing 24% crude protein and 54% carbohydrate between days 14 and 20. All diets contained 15% vitamin and mineral mixture, 4.7% fat, and 2.4% fiber (USDA-Feed Mill, Beltsville, MD). All experiments were approved by the Beltsville Agricultural Research Center Institutional Animal Care and Use Committee.

2.2. Measurement of body weights and gut lesion scores

Body weights were measured at day 20 post-hatch (day 6 post-infection with *E. maxima* and day 2 post-infection with *C. perfringens*) as described (Lee et al., 2011). For gut lesion scoring, two 10 cm sections of intestine flanking the diverticulum were harvested from 5 chickens per group at day 20 post-hatch and evaluated on a scale from 0 (no lesions) to 4 (severe lesions) in a blinded fashion by 3 independent observers as described (Prescott, 1979).

2.3. PCR amplification and pyrotag sequencing

Total genomic DNA was extracted from the contents of the intestinal ileum using the UltraClean Fecal DNA Kit (MO BIO Laboratories, Carlsbad, CA) and stored at −20 °C. The variable region 3 to 5 (V3–V5) of the bacterial 16S rRNA gene was amplified using the forward primer 341F (5′-ACTCCTACGGGAGGCAGCAG-3′) and the reverse primer 926R

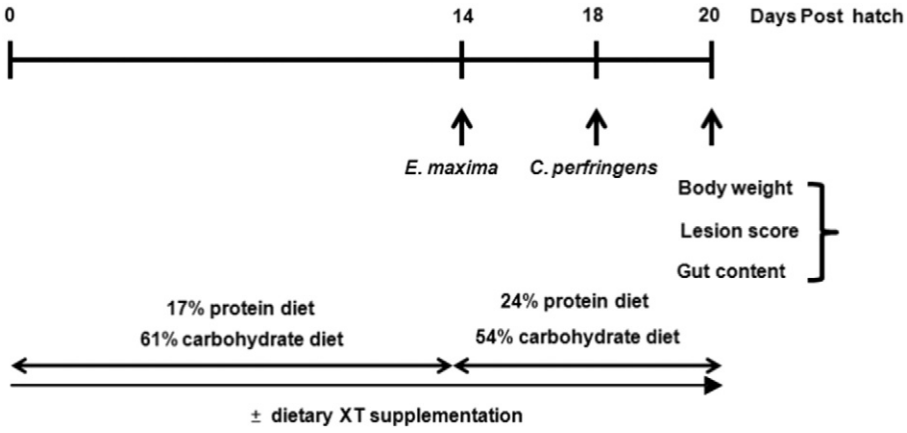

Fig. 1. Schematic illustration of the experimental design. One-day-old Cobb, Hubbard, and Ross chickens were fed a standard unsupplemented diet or a diet supplemented with a mixture of *Capsicum*/*Curcuma longa* oleoresins (XT). The diet contained 17% protein and 61% carbohydrate between days 1 and 14 and 24% protein and 54% carbohydrate between days 15 and 20. Chickens were infected by oral gavage with *E. maxima* at day 14 and *C. perfringens* at day 18. At day 20, body weights and lesion scores were measured and the intestinal ileum was sampled for microbiome analysis.

(5′-CCGTCAATTCMTTTGAGTTT) as described (Hume et al., 2011). Primers contained multiplex identifier sequences (barcodes) and appropriate adaptor sequences for pyrotag sequencing (http://oklbb.ezbiocloud.net/content/1001). 0.09pt?>PCR amplifications were performed in a final volume of 50 μl with 10× Taq buffer, dNTP mixture, 10 μM of each primer, and 2 U of Taq polymerase (Takara, Shiga, Japan) using a Mastercycler gradient thermocycler. The following PCR profile was performed: 5 min at 94 °C, 30 s at 94 °C, 30 s at optimal annealing temperature 55 °C (30 cycles for first reaction and 25 cycles for nested PCR), and 30 s at 72 °C, followed by a final extension at 72 °C for 7 min. The PCR product was confirmed by 2% agarose gel electrophoresis and visualized under a Gel Doc image analysis system (BioRad, Hercules, CA). Amplified products were purified with the QIAquick PCR purification kit (Qiagen, Valencia, CA). Equal amounts of each amplicon from different samples in the same treatment group were pooled and subjected to a second round of purification using the AMPure bead kit (Agencourt Bioscience, Beverly, MA) followed by amplification on the sequencing beads by emulsion PCR. Recovered beads from emulsion PCR were deposited on a 454 Picotiter Plate and sequenced at Macrogen Korea (Seoul, Korea) with GS Junior system following the manufacturer's instructions (Roche, Branford, CT).

2.4. Sequence analysis

Pyrotag sequencing reads were separated by their barcodes and extracted from the data set. Sequencing reads containing two or more ambiguous nucleotides, low quality score (average score < 25), or reads shorter than 300 bp were discarded. The Mothur software package was used to analyze microbial communities (http://www.mothur.org/) (Schloss et al., 2009). CD-HIT-OTU was used to remove chimeric sequences (http://weizhong-lab.ucsd.edu/cd-hit-otu/) (Li et al., 2012). Sequences were assigned to groups based on their barcodes, and similar sequences were assigned into operational taxonomic units (OTUs) at a pairwise identify of 97% using UCLUST (http://www.drive5.com/usearch/). The most abundant sequence in each OTU, designated as the representative sequence, was classified taxonomically using Ribosomal Database Project (RDP) classifier 2.0.1 (Cole et al., 2009). The Silva rRNA database (http://www.arb-silva.de/) was used to identify regions of local similarity between sequences (Quast et al., 2013).

2.5. Statistical analysis

Body weight and lesion scores were subjected to one-way analysis of variance using SPSS 22.0 for Windows (SPSS Inc., Boston, MA). Mean values of treatment groups were compared using the t-test or the Duncan's multiple range test and differences were considered statistically significant at $P < 0.05$.

3. Results

3.1. Effects of dietary phytonutrients on body weights and gut lesion scores

Cobb, Hubbard, and Ross chickens co-infected with *E. maxima* and *C. perfringens* in an experimental disease model of avian NE had reduced body weights at day 20 post-hatch compared with uninfected birds of the same breed (Fig. 2A). Infected chickens given a dietary supplementation with the *Capsicum*/Curcuma *longa* oleoresin mixture had increased body weights compared with infected birds fed the unsupplemented diet. Dietary supplementation with the oleoresin mixture also decreased intestinal lesion scores of the three infected chicken breeds compared with the unsupplemented and infected controls (Fig. 2B).

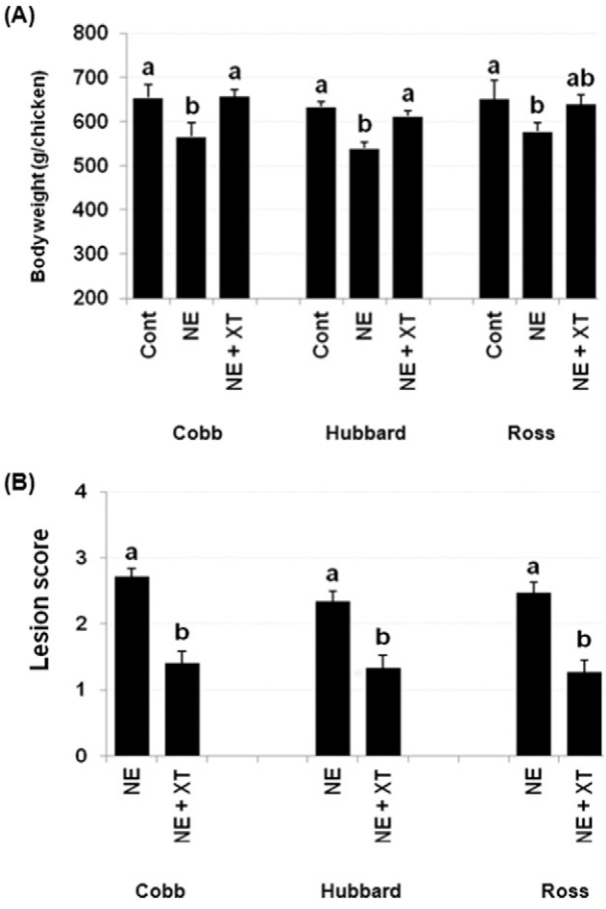

Fig. 2. Body weights and intestinal lesions in chickens during experimental NE. Cobb, Hubbard, and Ross chickens were fed an unsupplemented diet and uninfected (Cont), an unsupplemented diet and co-infected with *E. maxima* and *C. perfringens* (NE), or an oleoresin-supplemented diet and co-infected with *E. maxima* and *C. perfringens* (NE + XT). Body weights (A) and intestinal lesion scores (B) were measured at day 20. Each bar represents the mean ± SEM value (n = 15). Bars not sharing the same letter within each chicken breed are significantly different according to the Duncan's multiple range test ($P < 0.05$).

3.2. Global diversity of intestinal microbial communities across all treatment groups

A total of 49,031 16S rRNA sequences were generated from the contents of the intestinal ileum of unsupplemented/uninfected (control), unsupplemented/co-infected (NE), and oleoresin supplemented/co-infected (NE + XT) Cobb, Hubbard, and Ross chickens (Table 1). Individual pyrosequencing reads corresponding to specific OTUs were assigned to bacterial phyla and genera by homology comparison using the RDP and Silva databases. Table 2

Table 1
Summary of pyrotag sequencing reads and identified OTUs in the intestinal microbiota of three broiler breeds.

Breed	Treatment	Total sequencing reads	Total OTUs[a]
Cobb	Control	6388	62
	NE	1108	34
	NE + XT	10,030	186
Hubbard	Control	3745	66
	NE	6416	81
	NE + XT	4743	49
Ross	Control	7257	40
	NE	5156	43
	NE + XT	4188	51

[a] OTUs, operational taxonomic units.

Table 2
Taxonomic summary of the major OTUs identified across all treatment groups in three broiler breeds.

Breed	Phylum__Class__Order__Family__Genus__Species	GenBank Accession no.	Number of OTUs
Cobb (16)	Firmicutes__Bacilli__Lactobacillales__*Lactobacillaceae*__*Lactobacillus*__*Lactobacillus salivarius*	AY137587.1.1489	2
	Firmicutes__Bacilli__Lactobacillales__*Lactobacillaceae*__*Lactobacillus*__*Lactobacillus* phage Sal3	CP000233.1410454.1411970	9
	Firmicutes__Bacilli__Lactobacillales__*Lactobacillaceae*__*Lactobacillus*__uncultured bacterium	EU453809.1.1431	7
	Firmicutes__Bacilli__Lactobacillales__*Lactobacillaceae*__*Lactobacillus*__uncultured bacterium	GQ075083.1.1385	14
	Firmicutes__Bacilli__Lactobacillales__uncultured bacterium	FJ672018.1.1416	13
	Firmicutes__Bacilli__Lactobacillales__*Streptococcaceae*__*Streptococcus*__uncultured bacterium	EU778976.1.1408	4
	Firmicutes__Clostridia__Clostridiales__*Clostridiaceae*__*Candidatus* Arthromitus__unidentified bacterium	X80834.31.1471	1
	Firmicutes__Clostridia__Clostridiales__*Lachnospiraceae*__*incertae sedis*__uncultured bacterium	FJ369760.1.1358	11
	Firmicutes__Clostridia__Clostridiales__*Peptostreptococcaceae*__*incertae sedis*__uncultured bacterium	EU475172.1.1363	12
	Firmicutes__Clostridia__Halanaerobiales__*Halobacteroidaceae*__*Halobacteroides*__*Halobacteroidaceae* bacterium S200	FJ931099.1.1487	8
	Proteobacteria__Gammaproteobacteria__Acidithiobacillales__*Acidithiobacillaceae*__*Acidithiobacillus*__uncultured bacterium	EF695073.1.1513	10
	Proteobacteria__Gammaproteobacteria__Enterobacteriales__*Enterobacteriaceae*__*Escherichia-Shigella*__uncultured bacterium	EF604214.1.1499	16
	Proteobacteria__Betaproteobacteria__Burkholderiales__*Comamonadaceae*__*Variovorax*__uncultured bacterium	GQ246403.1.1498	6
	Bacteroidetes__Bacteroidia__Bacteroidales__*Bacteroidaceae*__*Bacteroides*__uncultured bacterium	GQ491320.1.1367	15
	Cyanobacteria__Chloroplast__Zea mays	X86563.95161.96651	3
	Cyanobacteria__Chloroplast__uncultured *Oscillatoria* spp.	EU341292.1.1459	5
Hubbard (8)	Firmicutes__Bacilli__Lactobacillales__*Lactobacillaceae*__*Lactobacillus*__uncultured bacterium	GQ075083.1.1385	6
	Firmicutes__Bacilli__Lactobacillales__*Lactobacillaceae*__*Lactobacillus*__uncultured bacterium	EU452227.1.1437	8
	Firmicutes__Bacilli__Lactobacillales__*Lactobacillaceae*__*Lactobacillus*__*Lactobacillus salivarius*	AY137587.1.1489	1
	Firmicutes__Bacilli__Lactobacillales__*Streptococcaceae*__*Streptococcus*__uncultured bacterium	EU779014.1.1408	4
	Firmicutes__Clostridia__Clostridiales__*Clostridiaceae*__*Candidatus* Arthromitus__unidentified bacterium	X87244.1.1441	7
	Firmicutes__Clostridia__Halanaerobiales__*Halobacteroidaceae*__*Halobacteroides*__*Halobacteroidaceae* bacterium S200	FJ931099.1.1487	3
	Proteobacteria__Betaproteobacteria__Burkholderiales__*Comamonadaceae*__*Variovorax*__uncultured bacterium	GQ246403.1.1498	2
	Cyanobacteria__Chloroplast__*Zea mays*	AY928077.95198.96680	5
Ross (15)	Firmicutes__Bacilli__Lactobacillales__*Lactobacillaceae*__*Lactobacillus*__uncultured bacterium	GQ070754.1.1388	8
	Firmicutes__Bacilli__Lactobacillales__*Streptococcaceae*__*Streptococcus*__uncultured bacterium	EU778976.1.1408	3
	Firmicutes__Bacilli__Lactobacillales__*Lactobacillaceae*__*Lactobacillus*__uncultured bacterium	EU453809.1.1431	5
	Firmicutes__Bacilli__Lactobacillales__*Lactobacillaceae*__*Lactobacillus*__*Lactobacillus* phage Sal3	CP000233.1410454.1411970	7
	Firmicutes__Bacilli__Lactobacillales__*Streptococcaceae*__*Streptococcus*__*Streptococcus gallolyticus* subsp. *macedonicus*	Z94012.1.1542	14
	Firmicutes__Clostridia__Clostridiales__*Clostridiaceae*__*Clostridium*__uncultured bacterium	GU455313.1.1479	9
	Firmicutes__Clostridia__Clostridiales__*Clostridiaceae*__*Candidatus* Arthromitus__unidentified bacterium	X87244.1.1441	15
	Firmicutes__Clostridia__Clostridiales__*Lachnospiraceae*__*incertae sedis*__bacterium ic1291	DQ057456.1.1454	12
	Firmicutes__Clostridia__Clostridiales__*Lachnospiraceae*__uncultured__uncultured bacterium	DQ057421.1.1460	6
	Firmicutes__Clostridia__Clostridiales__*Veillonellaceae*__*Veillonella*__uncultured bacterium	EU774834.1.1329	10
	Firmicutes__Clostridia__Halanaerobiales__*Halobacteroidaceae*__*Halobacteroides*__*Halobacteroidaceae* bacterium S200	FJ931099.1.1487	2
	Proteobacteria__Betaproteobacteria__Burkholderiales__*Comamonadaceae*__*Variovorax*__uncultured bacterium	GQ246403.1.1498	4
	Cyanobacteria__Chloroplast__*Zea mays*	AY928077.95198.96680	13
	Cyanobacteria__Chloroplast__uncultured bacterium	DQ532257.1.1463	1
	Actinobacteria__Actinobacteria__Actinobacteridae__Actinomycetales__Micrococcineae__*Dermabacteraceae*__*Brachybacterium*__*Brachybacterium faecium*	X91032.1.1513	11

( ), number of unique major OTUs.
incertae sedis, uncertain placement.

summarizes the taxonomic classifications of the major OTUs identified in all treatment groups of the three chicken breeds. Sixteen major OTUs were identified in Cobb, 8 OTUs in Hubbard, and 15 OTUs in Ross. Firmicutes, Proteobacteria, and Cyanobacteria were the most common phyla detected in all chickens, with greater than 97% of all sequencing reads identified as Firmicutes. Bacteroidetes was exclusively found in Cobb chickens and Actinobacteria was restricted to Ross. At the genus level, Hubbard chickens exhibited a fewer number of bacterial genera (n = 5) compared with Cobb (n = 9) and Ross (n = 9) birds. Bacterial beta diversity was determined by molecular phylogenetic analysis using the maximum likelihood method. Beta diversity at the phylum level among the combined treatment groups for Cobb, Hubbard, and Ross chickens is illustrated in Fig. 3. Beta diversity at the OTU level in the three broiler breeds in the separate treatment groups is illustrated in Fig. 4. Included in this analysis were unsupplemented chickens infected with either *C. perfringens* (CP) alone or *E. maxima* (EM) alone. In Cobb chickens, OTU diversity was relatively similar in the control, CP, NE, and NE + XT groups, while the EM group exhibited a dissimilar degree of diversity (Fig. 4). In Hubbard chickens, OTU diversity was comparable in the control, EM, CP, and NE + XT groups, while the NE group exhibited a relatively unique level of diversity. In Ross chickens, OTU diversity was similar in the control, EM, CP, and NE groups, while the NE + XT group exhibited a different extent of diversity.

3.3. Comparisons of intestinal microbiomes in the control and NE treatment groups

Compared with the control group, the total number of OTUs was decreased in the NE group of Cobb chickens (34 *vs.* 62), and increased in the NE groups of Hubbard (81 *vs.* 66) and Ross (43 *vs.* 40) birds (Table 1). Fig. 5 summarizes the relative distribution of the predominant bacterial genera identified in the control and NE treatment groups of the 3 breeds. Compared with the control group, the NE group of Cobb chickens had increased percentages of *Lactobacillus* and *Selenihalanaerobacter*, and decreased fractions of *Candidatus* Arthromitus and *Streptococcus*. In Hubbard chickens, the NE group had decreased fractions of *Lactobacillus*, *C.* Arthromitus, and *Streptococcus*, and increased percentages of *Selenihalanaerobacter* and *Variovorax* compared with the control group. In Ross chickens, the NE group had decreased fractions of *Lactobacillus* and *C.* Arthromitus, and increased percentages of *Selenihalanaerobacter* and *Variovorax* compared with the control

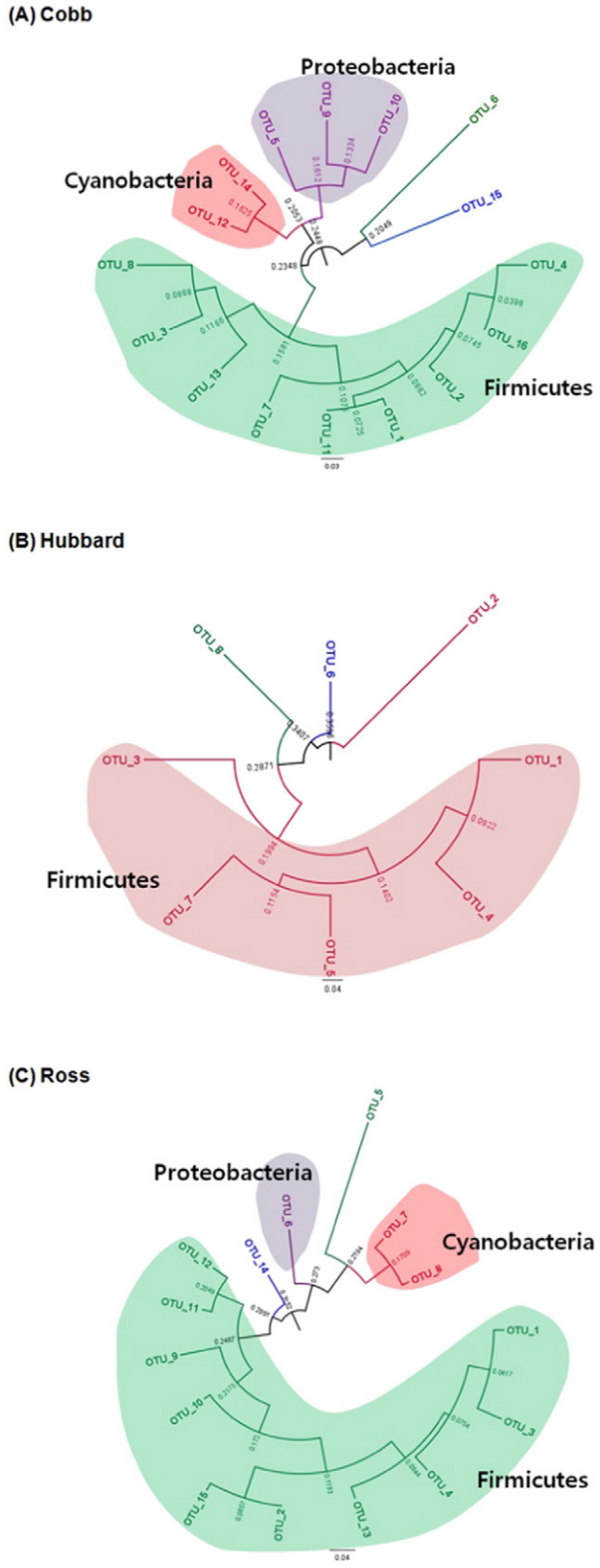

Fig. 3. Intestinal ileum bacterial beta diversity at the phylum level among all treatment groups. Major OTUs for Proteobacteria, Cyanobacteria, and Firmicutes illustrated in (A) Cobb, (B) Hubbard, and (C) Ross chickens.

group. Ross chickens in the control and NE groups were notable for their relatively high fractions of *Streptococcus* compared with Cobb and Hubbard birds.

3.4. Comparisons of intestinal microbiomes in the NE and NE + XT treatment groups

Compared with the NE group, the total number of OTUs was increased in the NE + XT group of Cobb (186 *vs.* 34) and Ross (51 *vs.* 43) chickens, and decreased in the NE + XT group of Hubbard (49 *vs.* 81) birds (Table 1). Compared with the NE group, the NE + XT group of Cobb chickens had an increased percentage of *Lactobacillus*, and decreased fractions of *Selenihalanaerobacter* and *Geitlerinema* (Fig. 5). In Hubbard chickens, the NE + XT group had increased percentages of *Lactobacillus* and *Streptococcus*, and decreased fractions of *Selenihalanaerobacter* and *Variovorax* compared with the NE group. In Ross chickens, the NE + XT group had increased percentages of *Selenihalanaerobacter*, *Clostridium*, *Calothrix*, and *Geitlerinema* and decreased fractions of *Lactobacillus*, *Streptococcus*, and *Variovorax* compared with the NE group.

3.5. Core OTUs in the control, NE, and NE + XT groups

The number and distribution of common and unique core OTUs in the control, NE, and NE + XT groups of the three broiler breeds are shown by Venn diagram in Fig. 6, and their taxonomic classifications are listed in Table 3. In the control group, *Halobacteroides* was identified in Cobb and Hubbard chickens, whereas *C.* Arthromitus was detected in Ross (Table 3A). In the NE group, only Hubbard broilers had a core OTU, *Lactobacillus* (Table 3B). In the NE + XT group, *Lachnospiraceae* (*incertae sedis)* and *Escherichia-Shigella* were detected in Cobb chickens, and *Veillonella* and *Dermabacteraceae* were seen in Ross birds (Table 3C). Finally, when the three treatment groups were analyzed in aggregate, the major OTUs identified were *C.* Arthromitus and *Lactobacillus* in Cobb and Hubbard chickens, and *Halobacteroides* and *Lactobacillus* in Ross birds (Table 4).

4. Discussion

Selective breeding programs of broiler chickens by poultry industry over 50 years have preferentially selected for economic traits such as growth rate, feed conversion and breast yields (Davis et al., 2011; Paxton et al., 2013; Zekarias et al., 2002). Nonetheless, genetic selection will be complicated by their instability according to chicken line, age and environment (Calenge and Beaumont, 2012). These efforts often led to increase disease susceptibility and less than optimal immune response to pathogens. Indeed, blunted humoral and cellular immune responses have been described in modern broiler breeds selected for high economic performance (Cheeseman et al., 2007; Hong et al., 2012; Jang et al., 2013) related, in part, to diminished baseline functional parameters of their mononuclear phagocytes (Qureshi and Miller, 1991). Given that innate immunity mediated by macrophages plays a protective role in the development and progression of avian necrotic enteritis (NE), it is not unreasonable to predict that poultry genetics also plays a major role in dictating the type of host response to NE. Until now, however, limited information has been available on the effect of commercial chicken breeds on NE susceptibility, which is an emerging and devastating poultry disease caused by *C. perfringens* infection, typically following *Eimeria*-induced intestinal damage (Lee et al., 2012; Williams, 2005).

Our laboratory previously observed that 3 commercial broiler breeds (Cobb, Hubbard and Ross) showed significant difference in disease susceptibility to NE (Jang et al., 2013). Three broiler breeds have been genetically selected by commercial breeding companies (Cobb-Vantress, Hubbard and Aviagen) to improve economic traits such as breast yield, feed efficiency and fast growth and mortality. There are comparative studies of economic traits and value of these broiler breeds (Gonzales et al., 1998; Jiang et al., 1998; Kapell et al., 2012; Olanrewaju et al., 2014). The present study investigated the breed differences in performance to experimental NE and correlating changes of intestinal bacterial communities in 3 commercial broiler breeds fed

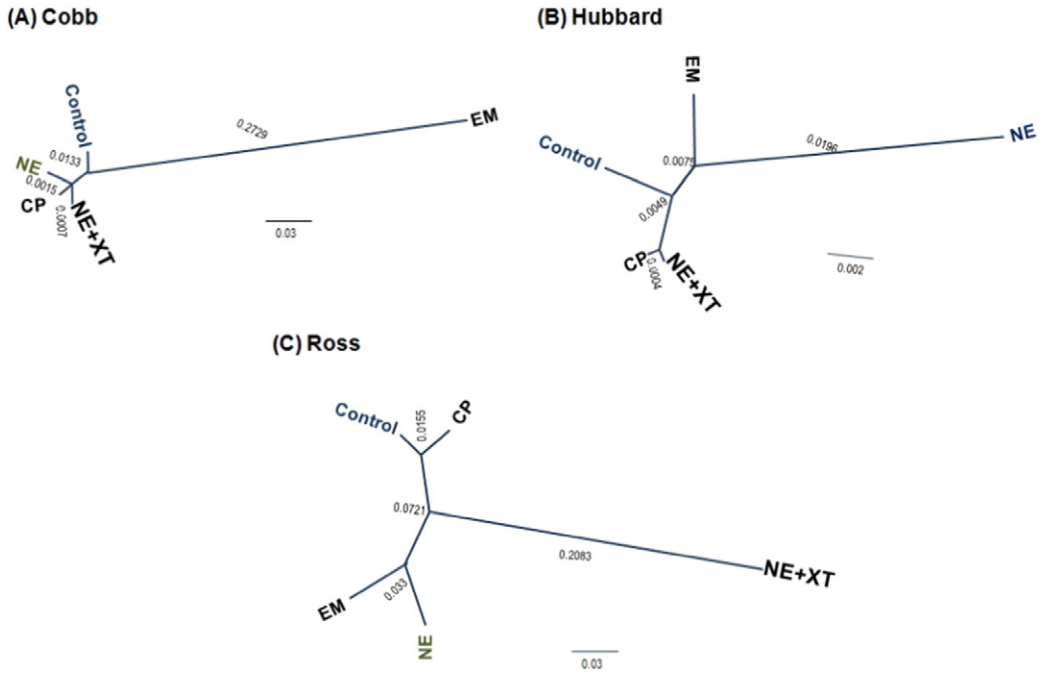

Fig. 4. Intestinal ileum bacterial beta diversity at the OTU level. (A) Cobb, (B) Hubbard, and (C) Ross chickens were unsupplemented and uninfected (Control), unsupplemented and *E. maxima*-infected (EM), unsupplemented and *C. perfringens*-infected (CP), unsupplemented and *E. maxima*/*C. perfringens* co-infected (NE), or oleoresin-supplemented and *E. maxima*/*C. perfringens* co-infected (NE + XT). Molecular phylogenetic analysis of 16S rRNA sequences was performed by the maximum likelihood method. The relative lengths of each line in the distance trees are indicated numerically, with longer line indicative of more dissimilar diversity.

dietary phytonutrients, *Capsicum*/*C. oleoresins* (XT). Because there was no previous comparison of gut microbiota changes in major 3 commercial broiler breeds, the focus of this study was to identify the differences of gut microbiota communities before and after XT supplements. Three groups per each breed were assigned: unsupplemented and uninfected (control), unsupplemented and *E. maxima*/*C. perfringens* co-infected (NE), or *Capsicum*/*Curcuma* oleoresin supplemented and co-infected (NE + XT). Compared with Hubbard and Ross chickens in this study, the Cobb breed was previously observed to exhibit greater body weight loss between days 14 and 20 (days 0 and 6 post-infection with *E. maxima*), and greater gut lesions at day 20, using the same co-infection model of avian NE used in the current study When comparing the NE treatment group with uninfected controls, we observed that Cobb and Hubbard chickens had significantly increased body weight loss (90 ± 10 g) at day 20, which was significantly greater than that of Ross birds (70 ± 7.0 g). Further, Cobb chickens in the NE treatment group had a greater lesion score (2.8 ± 0.1) at day 20 compared with NE-treated Hubbard (2.3 ± 0.1) and Ross (2.4 ± 0.1). These combined data suggest that the Cobb breed is more susceptible to experimental NE compared with Hubbard or Ross. In spite of these interbreed differences, dietary supplementation with the oleoresin mixture in the NE + XT treatment group restored body weights as seen in the unsupplemented/uninfected controls in all 3 broiler breeds (Fig. 2A, $P > 0.05$), and treatment groups showed significantly higher ($P < 0.05$) gut lesion scores than controls in all breeds (Fig. 2B). The results clearly demonstrated in showing genetic as well as dietary phytonutrient effects on NE response in three commercial broiler breeds.

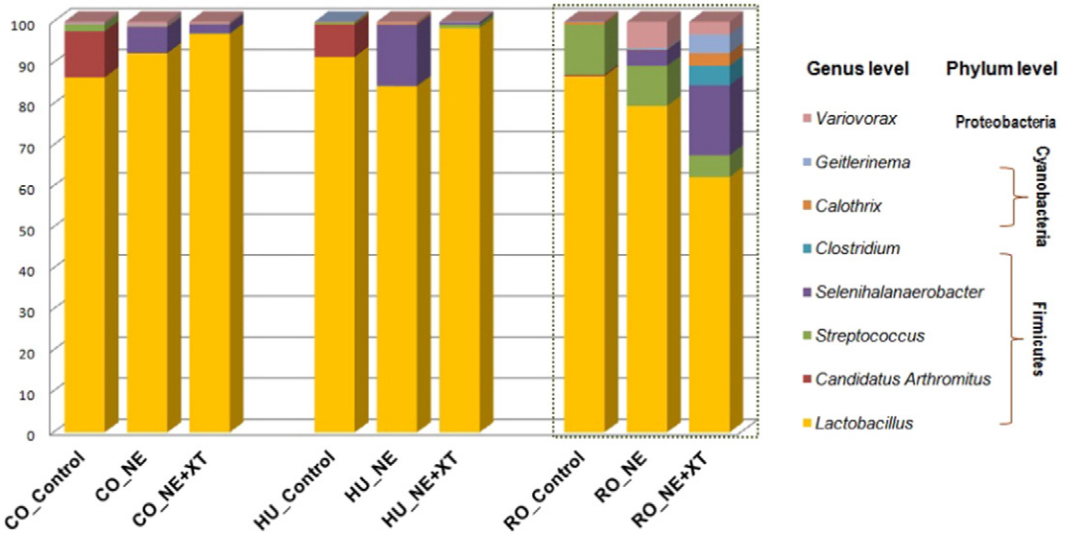

Fig. 5. Comparison of bacterial composition and diversity in three commercial broiler breeds. Cobb (CO), Hubbard (HU), and Ross (RO) chickens in the control, NE, and NE + XT treatment groups were compared at phylum and genus levels.

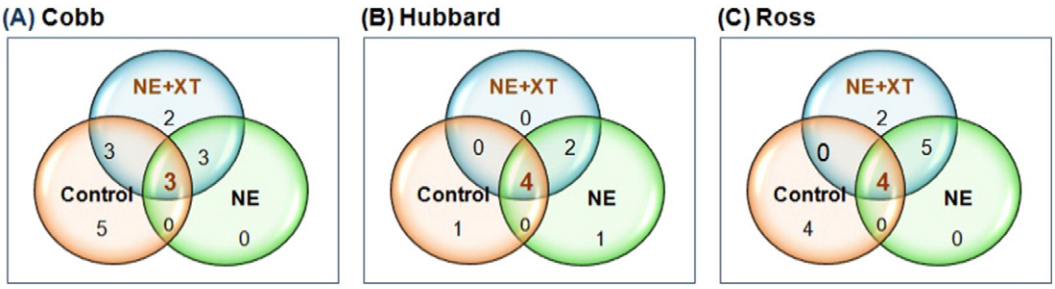

Fig. 6. Core OTUs among NE + XT, NE and control groups in three commercial broiler breeds. Venn diagram illustrated the number of common and unique core OTUs among the control, NE, and NE + XT treatment groups of (A) Cobb, (B) Hubbard, and (C) Ross chickens.

Given the shorter digestive tract and faster digesta transit in poultry than in mammalian animals, the average whole tract transit time in poultry tract is less than 3.5 h. Gut microbiota communities in small intestine (duodenum, jejunum and ileum) are less abundant than cecum (Hughes, 2008; Pan and Yu, 2014). The advent of high-throughput DNA sequencing technologies has allowed an integrative approach to identify the bacteria communities residing within or on their host organism. This study showed that Firmicutes is the dominant phylum and *Lactobacillus* was the predominant bacterial genus identified in the intestinal ileum in all broiler breeds and all treatment groups. This result is consistent with previous studies that showed *Lactobacillus* as the principal microorganism in the gastrointestinal tract of uninfected conventional broilers (Gong et al., 2007; Nakphaichit et al., 2011). Because Firmicute is fat loving gram positive bacteria (Fukuda and Ohno, 2014; Jain and Walker, 2014; Ravussin et al., 2012), this result suggests interrelationship of this bacteria and genetic selection for fast growing characteristics of these broilers by the industry. In addition, we observed that the relative changes in the proportion of intestinal *lactobacilli*, as well as the total number of OTUs, differed between the broiler breeds. When comparing the control and NE treatment groups, both parameters correlated with breed susceptibility or resistance to experimental NE. Thus, the more NE susceptible Cobb breed had a decreased number of OTUs and an increased fraction of *Lactobacillus*, while the more NE resistant Hubbard and Ross chickens had the opposite pattern, *i.e.* an increase in OTUs and decreased fraction of *Lactobacillus*. This relationship, however, was not seen when comparing the NE and NE + XT treatment groups, where OTUs were increased in Cobb and Ross but decreased in Hubbard, and *Lactobacillus* was increased in Cobb and Hubbard, but decreased in Ross.

There is lack of studies of a consistent pattern of gut microbiome alterations during experimental NE in the chicken. At least two studies reported that the fraction of intestinal *Lactobacillus* was equal irrespective of NE disease status (Jia et al., 2009; Mikkelsen et al., 2009). By contrast, the previous study (Liu et al., 2012) reported that *lactobacilli* were increased in the gut of Arbor Acre breed broilers with experimental NE compared with uninfected controls. Similarly, an increased population of *Escherichia coli* was seen in the gut of birds with experimental NE compared with uninfected controls (Ooi et al., 1975). Finally, the number of *C. perfringens* isolated from the intestine of chickens with experimental NE was negatively correlated with the total population of *Lactobacillus* (Rychlik et al., 2014). *Lactobacillus crispatus*, *Lactobacillus salivarius*, and *Lactobacillus reuteri* all have been associated with increased protective immunity, greater inflammation, and heightened antimicrobial activity against pathogenic intestinal microbes in birds and mammals (Liu et al., 2012; Nakphaichit et al., 2011; Tobita et al., 2010).

In the present study, *C.* Arthromitus known as segmented filamentous bacteria (SFB), noncultivable, spore-forming, *Clostridia*-related commensal bacteria that colonizes the digestive tracts of animal species, was identified in 3 commercial broiler breeds (Snel et al., 1995; Thompson et al., 2012). As the core OTU, *C.* Arthromitus was identified in all three groups (control, NE and NE + XT) of the Cobb and Hubbard broilers. In Ross broilers, it was detected only in the control group and not found in NE and (NE + XT) samples (Tables 2 and 3). It is assumed that these bacteria may move up and down to other intestinal segments after infection. The most intriguing feature of SFB is their close interaction with epithelial cells in the terminal ileum and their intimate cross talk with the host immune system. *C.* Arthromitus is belonging

Table 3
Taxonomic summary of the major OTUs identified for each treatment group in three broiler breeds.

	Phylum	Class	Order	Family	Genus	Accession number
A. Control group (OTUs)						
Cobb (5)	Firmicutes	Clostridia	Halanaerobiales	*Halobacteroidaceae*	*Halobacteroides*	FJ931099.1.1487
		Bacilli	Lactobacillales	*Lactobacillaceae*	*Lactobacillus*	EU453809.1.1431
						GQ075083.1.1385
	Bacteroidetes	Bacteroidia	Bacteroidales	*Bacteroidaceae*	*Halobacteroides*	GQ491320.1.1367
	Cyanobacteria	Chloroplast			*Zea mays*	X86563.95161.96651
Hubbard (1)	Firmicutes	Clostridia	Halanaerobiales	*Halobacteroidaceae*	*Halobacteroides*	FJ931099.1.1487
Ross (4)	Firmicutes	Clostridia	Clostridiales	*Clostridiaceae*	*Clostridium*	GU455313.1.1479
				Clostridiaceae	*Candidatus Arthromitus*	X87244.1.1441
				Lachnospiraceae	*incertae sedis*	DQ057456.1.1454
		Bacilli	Lactobacillales	*Streptococcaceae*	*Streptococcus*	Z94012.1.1542
B. NE group (OTUs)						
Hubbard (1)	Firmicutes	Bacilli	Lactobacillales	*Lactobacillaceae*	*Lactobacillus*	EU452227.1.1437
C. NE + XT Group (OTUs)						
Cobb (2)	Firmicutes	Clostridia	Clostridiales	*Lachnospiraceae*	*incertae sedis*	FJ369760.1.1358
	Proteobacteria	Gammaproteobacteria	Enterobacteriales	*Enterobacteriaceae*	*Escherichia-Shigella*	EF604214.1.1499
Ross (2)	Firmicutes	Clostridia	Clostridiales	*Veillonellaceae*	*Veillonella*	EU774834.1.1329
	Actinobacteria	Actinobacteridae	Actinomycetales	*Micrococcineae*	*Dermabacteraceae*	X91032.1.1513

Table 4
Core OTUs (control + NE + NE/XT) in three broiler breeds.

	Phylum	Class	Order	Family	Genus	Accession number
Cobb (4)	Firmicutes	Clostridia	Clostridiales	*Clostridiaceae*	*Candidatus* Arthromitus	X80834.31.1471
		Bacilli	Lactobacillales	*Lactobacillaceae*	*Lactobacillus*	GQ075083.1.1385
	Cyanobacteria					EU341292.1.1459
Hubbard (3)	Firmicutes	Clostridia	Clostridiales	*Clostridiaceae*	*Candidatus* Arthromitus	X87244.1.1441
		Bacilli	Lactobacillales	*Lactobacillaceae*	*Lactobacillus*	AY137587.1.1489
			Lactobacillales	*Lactobacillaceae*	*Lactobacillus*	GQ075083.1.1385
	Cyanobacteria	Chloroplast	Zea mays			AY928077.95198.96680
Ross (4)	Firmicutes	Clostridia	Halanaerobiales	*Halobacteroidaceae*	*Halobacteroides*	FJ931099.1.1487
		Bacilli	Lactobacillales	*Lactobacillaceae*	*Lactobacillus*	CP000233.1410454.1411970
			Lactobacillales	*Lactobacillaceae*	*Lactobacillus*	GQ070754.1.1388
	Cyanobacteria	Chloroplast				DQ532257.1.1463

to gut-indigenous *Clostridium* to develop and induce immune regulatory T (Treg) cells. Intestinal Treg cells are reported to express T cell receptors that recognize antigen derived from gut microbiota (Ivanov and Littman, 2010; Nagano et al., 2012; Suzuki et al., 2004). The recent study showed that SFB sends signals to control the balance between IL-17 producing T helper (Th17) cells which sustain mucosal immune, and Foxp3 + in the intestine (Ivanov and Littman, 2010) (Ivanov and Littman, 2010), Our previous studies also reported that the chicken IL-17A transcript increases in the duodenum and jejunum of *E. maxima* infected chickens (Kim et al., 2012, 2014). In this current study, upon XT treatment there was a different shift of bacterial community in samples of NE and (NE + XT) groups in all broiler breeds. Therefore, the co-infection of *E. maxima* and *C. perfringens* may influence to the presence of *C.* Arthromitus and host immune system in Ross chickens. It is interesting to observe that both Cobb and Hubbard breeds showed very similar pattern of bacterial changes. We presumed that Cobb and Hubbard may have same genetic background not Ross. It still remains crucial for investigating to be elucidated the functional immune modulatory role of *C.* Arthromitus on the dietary phytonutrients in genetically different broiler breeds. In the present study, despite of lower sequencing coverage (Table 1), the majority of the bacterial diversity was enable to be captured and identified (Table 2). However, it is necessary to evaluate the detail composition of gut microbiota community by sufficient sequencing depth and extensive interaction between members of gut microbiota and phytonutrient supplements on both NE infection and absent infection because of a large number of bacteria remain unidentified.

The major findings from the current study are (1) Cobb, Hubbard, and Ross chickens in the NE + XT treatment group had increased body weights and reduced intestinal lesions compared with the NE treatment group, (2) Cobb and Hubbard chickens in the NE + XT treatment group had an increased fraction intestinal *Lactobacillus* and/or *Streptococcus*, and decreased *Selenihalanaerobacter*, *Geitlerinema*, and/or *Variovorax* compared with the NE group, while (3) Ross chickens in the NE + XT group had decreased percentages of *Lactobacillus*, *Streptococcus*, and *Variovorax* and increased *Selenihalanaerobacter, Clostridium*, *Calothrix,* and *Geitlerinema* compared with the NE group. Interestingly, the Lactobacillus and Streptococcus that were increased in NE + XT treated Cobb and Hubbard broilers were decreased in Rossbirds, while the opposite was true for *Selenihalanaerobacter* and *Geitlerinema* that were decreased in NE + XT treated Cobb and Hubbard but increased in Ross.

In conclusion, we observed that dietary *Capsicum* and *C. longa* oleoresins regulate susceptibility to experimental avian NE and alter the intestinal microbiota of commercial broiler chickens. This study demonstrates that dietary phytonutrients exert beneficial effects on gut health to reduce the negative consequences of NE and nutratherapeutic mechanism may involve altering gut microbial communities. These new findings increase our understanding of 1) the positive effect of dietary phytonutrient as alternatives to antibiotics, 2) host genetics on the interaction of host–pathogen in NE and 3) the possible role of gut microbiota in local immune regulation in broiler chickens. This study is the first to report on the effects of dietary phytonutrients on gut microbiota in commercial broiler breeds and opens a new way to reduce the use of antibiotics in poultry disease control. Future studies on the role of the avian intestinal microbiome in immune regulation and host–pathogen interactions are expected to shed new light on the host response to NE that will be beneficial for practical poultry husbandry.

Conflict of interests

DB is a collaborator of InVivo ANH, France, mother company of Pancosma, Geneva, Switzerland that supplied XTRACT® Nature.

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